The Fine Structure

of the Nervous System of *Bithynia tentaculata* (Prosobranchia)

in Relation to Possible Neurosecretory Activity

BY

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(3 Plates; 2 Text figures)

INTRODUCTION

IT HAS BEEN SHOWN that several types of cells occur in the nervous system of *Bithynia tentaculata* (Linnaeus, 1758) which differ from the majority of neurones both in cytological detail and in their reactions to those stains used to detect neurosecretion (ANDREWS, 1968). They contain variable numbers of secretory droplets, and occur in groups in all ganglia except the pedal and osphradial, as well as in the visceral loop and visceral nerves. The following description of the fine structure of the nervous system in mature snails, fixed at a time of year when they are not breeding, is primarily concerned with examining the possibility that they are neurosecretory cells.

METHODS

The snails were collected in October and kept in tap water at room temperature. The nervous system was dissected and the surrounding connective tissue removed in cold 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2 - 7.4. Individual ganglia and short pieces of nerves were fixed in this solution for 2 hours, followed by 30 minutes in 1% osmium tetroxide in 0.1M veronal acetate buffer. The material was embedded in Epon 812, and sections were mounted on uncoated copper grids, stained in 5% uranyl acetate (WATSON, 1958), and lead citrate (REYNOLDS, 1963). They were examined in an AEI EM6B electron microscope.

RESULTS

The histological and ultrastructural features of the various types of cells found in the nervous system are summarized in Table 1, and their fine structure is illustrated diagrammatically in Figures 1 and 2.

Four types of cells were identified in histological preparations and described fully in an earlier paper (ANDREWS, 1968): so-called "ordinary" neurones, which are not stained selectively by any of the methods used (Figure 1, a, b); and 3 other types (S1, S2, S3), possibly neurosecretory, which are differentially stained. The possible neurosecretory cells include: one type restricted to the cerebral ganglia, which is distinctive in its strong affinity for phloxin (Figure 1c); a second, occurring in localized areas of all ganglia except the pedal and osphradial, and stained intensely by aldehyde fuchsin (Figures 2, a, b); and a third type, the "bipolar neurones" of the visceral connectives and nerves, also stained by aldehyde fuchsin (Figure 2c).

A study of the fine structure of the nervous system has revealed a more complex organization than this in the following respects:

(1) Secretory granules are present in the cytoplasm of "ordinary" neurones, as well as in possible neurosecretory cells, although they differ from neurosecretory granules both in size and number (Figures 1 and 2).

(2) The neurosecretory areas of the cerebral ganglia contain not one(as previously thought), but two types of neurosecretory cells, the one being phloxinophil (S1, Fig-
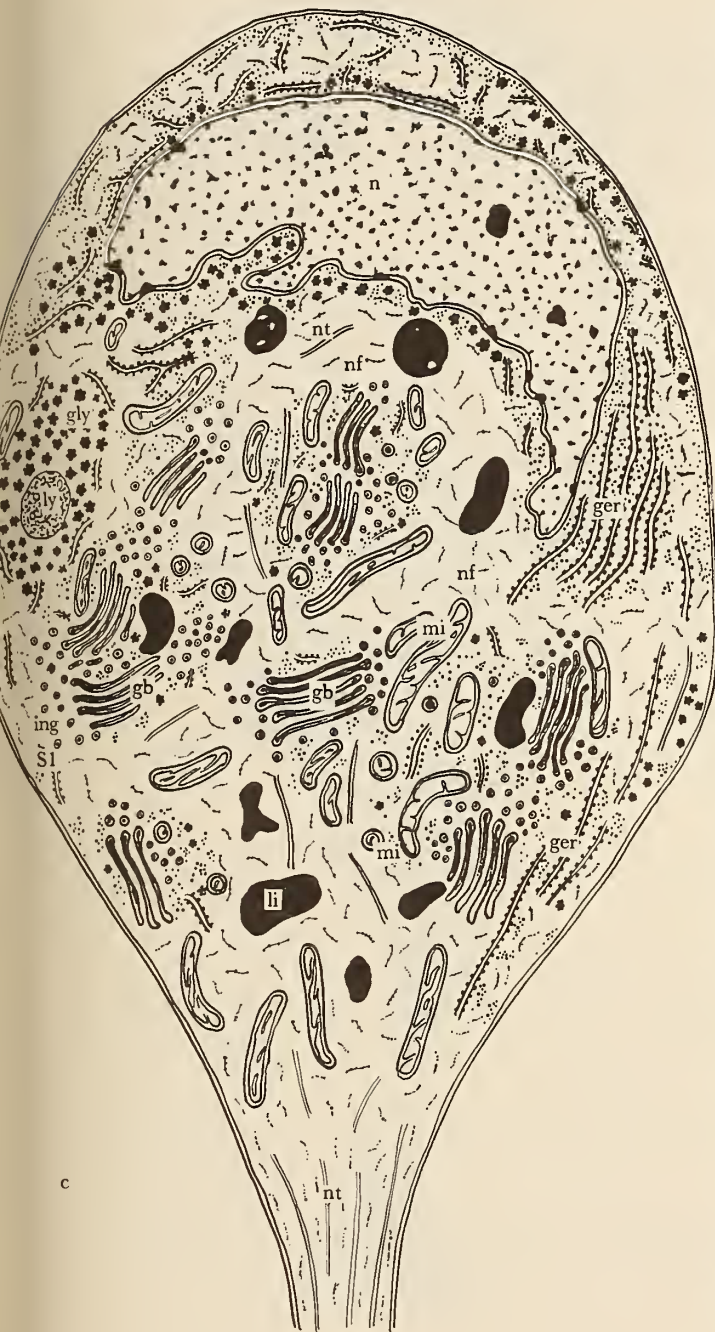
Figure 1

(a) An "ordinary" neurone, containing large numbers of yellow spherules and electron-dense secretory granules.

(b) An "ordinary" neurone with large numbers of yellowish-green spherules (lysosomes). Smooth endoplasmic reticulum is distended and filled with finely granular contents. The electron-dense secretory granules are accompanied by larger clear vesicles.

c – membrane-bounded inclusions thought to contain carotenoid pigment
cv – clear vesicle   gb – Golgi body
ger – granular endoplasmic reticulum   gly – glycogen
ing – immature neurosecretory granule   li – lipid
ly – lysosome (= inclusion containing lipofuscin pigment)
mi – mitochondrion   n – nucleus   nf – neurofibril
nt – neurotubule   r – ribosome   sg – secretory granule
ser – smooth endoplasmic reticulum

(c) An NS cell type S1. The cytoplasm is less dense than that of other neurones and neurofibrils are more conspicuous. Golgi bodies are numerous and endoplasmic reticulum and glycogen are localized.

ure 1c), the other fuchsinophil (S2, Figure 2a). This explains previously confusing anomalies in staining reactions.

(3) Two types of possible neurosecretory cells, indistinguishable by light microscopy, occur in other ganglia, and can be seen to have elementary neurosecretory granules of different sizes (S2 and S3, Figures 2, a, b).

(4) The "bipolar neurones" of the visceral connectives and nerves resemble mucus-secreting cells rather than neurones in their fine structure, and are probably some kind of neuroglial or connective tissue cell (S4, Figure 2c). Similar cells have been identified in electron micrographs of the neurosecretory areas of the ganglia, with the exception of the cerebral, but in sections of the ganglia they have been cut in such a plane that they look like narrow unipolar cells. In the nerves they are often cut longitudinally, and so look spindle-shaped.

Cell types S1, S2, and S3 will be referred to subsequently as NS cells, groups of them as NS areas, and neurosecretory material as NSM.

The neurones and neuroglial cells of *Bithynia* contain a variety of cytoplasmic inclusions, some of which may mimic NSM in routine histological preparations. For this reason it was important to confirm the identification of both these inclusions and NSM at the ultrastructural level. It was established that there are 3 categories of inclusions which take the form of cytoplasmic droplets or granules, and their characteristics are summarized in Table 1. Droplets of yellow carotenoid pigment (1) abound in "ordinary" neurones only; the greenish-yellow lipofuscins (2) are found in all cells, and in electron micrographs they have the appearance of lysosomes; the colourless droplets (3) of living cells are shown to be lipids, free in the cytoplasm.

The "ordinary" neurones of *Bithynia* have very dense cytoplasm (Figure 1a, b; Plate Figures *3, 4*), with granular endoplasmic reticulum (ger), free ribosomes (r), and neurotubules (nt), well developed peripherally. The central region of the cytoplasm is packed with long mitochondria (m), and Golgi bodies (gb). They may contain relatively small areas of glycogen (gly).

Most "ordinary" neurones have large numbers of droplets (c) containing yellow carotenoid pigment which appear in electron micrographs as areas of fine dense granules amongst a slightly less dense matrix, and surrounded by a unit membrane. Their sizes range from 0.2 - 1.0 μm. Larger inclusions (ly), some 1.0 - 2.0 μm, have much more variable contents composed of some electron-dense bodies in a finely granular ground substance of moderate density. Rarely, they contain traces of membranes. They bear a striking resemblance to the lysosomes of other cells, and are the lipofuscin spherules identified

Table 1

Characteristics of cytoplasmic inclusions and secretory granules of cells in the nervous system of *Bithynia tentaculata*

Staining Reaction	"Ordinary" neurones	NS cells S 1	NS cells S 2 & S 3	Mucoid cells S 4
Living, unstained	1. yellow < 1μm 2. yellow-green < 2μm 3. colourless, variable	bluish-white	bluish-white	bluish-white
Living, methylene blue	1. − 2. + 3. −	−	−	−
Iron haematoxylin	1. − 2. + 3. −	+	+	pale grey
Herlant	1. − 2. green 3. −	pale blue, vacuolated	purplish blue	intense blue
Azan	1. − 2. yellow green 3. −	pale blue, red clumps, vacuolated	red or blue, not vacuolated	intense blue
After oxidation: Chrome haematoxylin	1. − 2. + 3. −	−	+	pale grey
Phloxin	−	+	−	−
Aldehyde fuchsin	1. − 2. + 3. −	−	+	+
Orange G	−	+	−	−
Alcian blue	1. − 2. + 3. −	−	+	+
Alcian blue/alcian yellow	1. − 2. turquoise 3. −	pale yellow	turquoise	turquoise - deep green
Without oxidation: Alcian blue	−	−	−	−
Aldehyde fuchsin	−	−	−	−
Epon sections, Toluidine blue (JEON, 1965)	1. greenish grey 2. dark green 3. dark blue	+	+	pale grey - dark blue
Electron micrographs	1. electron-dense vesicles 2. lysosomes 3. unbounded lipid. Granules: dense 90nm clear 140 nm synaptic vesicles: 60 nm	No mature granules found	S2, 142×234 nm S3, 104 nm	S4, 1μm

Figure 2

(a)   An NS cell type S2. The cytoplasm contains large neurosecretory granules variable in shape.

(b)   An NS cell type S3. The neurosecretory granules are small and associated with glycogen granules.

ae – axonal ending	bm – basement membrane	f – fibre
gb – Golgi body	ger – granular endoplasmic reticulum	
gly – glycogen	mi – mitochondrion	n – nucleus
ly – lysosome (= inclusion containg lipofuscin pigment)		
ng – neurosecretory granule	nt – neurotubule	r – ribosome
rm – ruptured membrane	s – secretion	sg – secretory granule
sw – swelling on endoplasmic reticulum	v – vesicle	
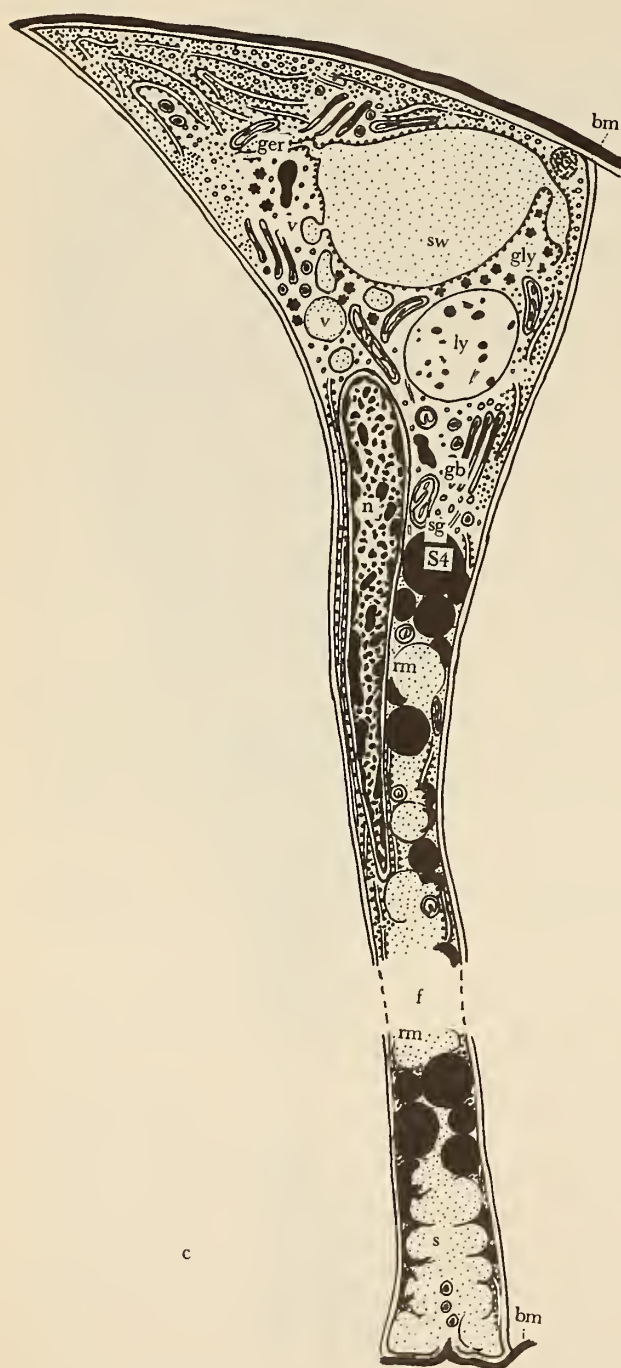
in histological preparations. Irregularly-shaped lipid droplets (li), without a limiting membrane, correspond to the colourless inclusions.

Two types of secretory granules have been found in the cytoplasm of the "ordinary" neurones, one type being electron-dense and approximately 90 nm in diameter (Plate Figures *3, 4,* and *14,* sg), the other, about 140 nm, with sparse contents of low electron density (Plate Figures *4* and *14,* v). The former occur throughout the cytoplasm, and seem to originate from the Golgi bodies, since clusters of them are found in these regions. In some cells they occur together with the larger granules, which never occur alone and seem to be associated with well developed smooth endoplasmic reticulum.

The phloxinophil NS cells (S1) of the cerebral ganglia (Plate Figure *5*) have less dense cytoplasm than any other neurones in *Bithynia* and in the snails examined they contain large numbers of immature granules near the prominent Golgi bodies, and very few mature neurosecretory granules. Stacks of granular endoplasmic reticulum surround the indented nucleus, but other areas of cytoplasm contain little other than neurofibrils (nf). Deposits of glycogen (gly) are much more extensive in the peripheral than in the central cytoplasm, often associated with lysosomes (ly), and mitochondria (mi) are more numerous centrally.

The second type of NS cell (S2), which is fuchsinophil, contains neurosecretory granules (ng) which often look dumb-bell-shaped, and have an average size of 142 by 234 nm (Figure *13*). The granular endoplasmic reticulum is well developed, and tends to lie parallel with the long axis of the neurone. Neurotubules are evident peripherally, whilst mitochondria and Golgi bodies are concentrated in the central cytoplasm. It is possible that small neurosecretory granules, 83 nm in diameter, arising from the Golgi bodies, fuse to form larger ones. In these specimens, cells of this type located in the cerebral ganglia contain fewer neurosecretory granules than those of other ganglia, and the Golgi bodies do not seem to be active. This suggests that they may be in a resting condition.

The other type of fuchsinophil NS cell (S3), is distinguished from the former (S2) by its smaller, spherical neurosecretory granules, which have an average diameter of 104 nm (Figures *11, 12*). The peripheral cytoplasm contains large areas of glycogen, which are absent in type S2. They are surrounded by densely packed granular endoplasmic reticulum (ger). If there are few neurosecretory granules present, they lie close to the cisternae of the endoplasmic reticulum (Figure *13*), but if there are many, they lie in the areas of glycogen deposits, and eventually completely fill them.

(c) A mucoid cell type S4, as seen in a section of a ganglion. The granular endoplasmic reticulum develops large swellings in the early stages of secretion. Secretory granules are much larger than neurosecretory granules.

Axones containing this type of NSM are found in the neuropile, and their endings are frequently packed with granules, together with an occasional lysosome. Other workers have reported finding small vesicles amongst larger secretory granules (AMOROSO, BAXTER, CHIQUOINE & NISBET, 1964, in *Archachatina,* and BOER, DOUMA & KOKSMA, 1968, in *Lymnaea*), but none has been found in *Bithynia*. There are indications that NSM is released at the axonal endings, in that the membranes of the granules are disrupted and their contents become less dense (Figure 12). In histological preparations the axonal endings of NS cells seem to be packed closely together in neurohaemal areas, as in *Lymnaea stagnalis* (BOER, 1965). However, electron micrographs reveal that they are interspersed amongst the fibres of the fourth type of secretory cell (S4), which are not neurones. For this reason one cannot make a quantitative estimate of NSM from paraffin sections.

The secretion of cells belonging to this last category (S4) has staining reactions very similar to those of NS cells, being glycoprotein in nature. In some preparations the alcian blue and alcian green method for distinguishing between different acidic groups (RAVETTO, 1960) stains these cells a deep green following oxidation, as compared with the paler turquoise of the NS cells, but if smaller quantities of secretion are present the cytoplasm is yellow, with turquoise secretory droplets, similar to the NS cells.

The cells are somewhat spindle-shaped in surface view, giving rise to 2 main fibres, which lie against the perineurium for some distance before penetrating into the deeper parts of the neuropile. In the visceral connectives and nerves their long axes lie parallel with the direction of the nerve fibres, and the tissue of the nerves is so dense that processes smaller than the 2 main fibres given off by the cells are often undetectable. In sections of the ganglia the cells usually look club-shaped (Figure 2c). They are approximately 11.5 μm in diameter at their widest in these sections, this part being adjacent to the perineurium. The nuclei may be ovoid or cylindrical in shape, depending on the quantity of secretion in the cytoplasm (Figures 2c and 7). In a cell filled with secretion, the nucleus, which is always peripheral, seems to be compressed into a cylindrical form, extending into the narrower part of the cell. If there is little secretion present the cytoplasm is dense and easily confused with that of a neurone. The peripheral region is packed with granular endoplasmic reticulum, which is sparse in the central cytoplasm. This is filled with mitochondria, lipid inclusions, pigment droplets (lysosomes), Golgi bodies, and glycogen. Secretory granules with a diameter of some 80 nm are scattered throughout the central cytoplasm (Figure 6, sg), and often clustered around the Golgi bodies.

The granular endoplasmic reticulum of these cells is very conspicuous during the earlier stages of secretion, the cisternae becoming much more swollen than in NS cells. At first small swellings develop (Figure 6, sw), which are filled with a finely granular material. They increase in size, and possibly coalesce, until they almost fill the cytoplasm. The ribosomes become dissociated from certain parts of the membranes (fm), and there are indications that small vesicles filled with secretion are pinched off from the reticulum at such points (Figure 8, v). Eventually, the cytoplasm is filled with membrane-bounded vesicles amongst which are traces of granular endoplasmic reticulum (Figure 7). The material in the vesicles varies in electron-density, and this may be an artifact of fixation, caused by the rupture of the membranes surrounding some of the vesicles (rm). Some areas of cytoplasm contain patches of lighter granular secretion free in the cytoplasm amongst traces of torn membranes (rm). In the fibrous processes of these cells vesicles tend to remain intact and electron-dense peripherally, but are swollen or burst centrally (Figures 9, 10).

The fibres contain small mitochondria and tubules similar in diameter to neurotubules (Figure 7, tu). Their distal ends expand and are in direct contact with the perineurium. Pore-like structures occur at intervals in these regions; the basement membrane can be seen to dip into depressions of the cell-surface, and at these points the cell membrane is discontinuous (Figure 10, p). In some of the processes nearly all the secretory vesicles are intact, but in others they are disrupted, possibly allowing the secretion to escape into the blood spaces of the perineurium (Figure 9, bs).

Two other types of cells have been found in, or closely associated with, the nervous system; these are the neuroglial cells, and connective tissue cells referred to as sheath cells. Neuroglial cells, with ovoid nuclei and little perinuclear cytoplasm, are interspersed amongst the neurones and nerve fibres. Their fine processes ensheathe the neurones, but make little more than superficial indentations into the cytoplasm, unlike the extensive trophospongium of the larger neurones of pulmonates (BULLOCK & HORRIDGE, 1965). They are often laden with spherules of lipofuscin, and their branching fibres are both narrower and denser than nerve fibres. Granular endoplasmic reticulum and mitochondria predominate in the cytoplasm.

In the nerves, nerve fibres are arranged in bundles, ensheathed in neuroglial fibres. Bundles are packed so tightly together that there are very few spaces between them, and sometimes it is possible to see that such a space is continuous with a blood space in the perineurium. They often contain a coil of cell membrane, extending from the distal end of a fine neuroglial fibre, and it is possible that they are a means of increasing the surface area across which

exchange of material with the haemolymph might occur. There is no evidence of poor fixation in these nerves to suggest that they might be artifacts.

The second type of cell associated with the nervous system in *Bithynia* forms a network conspicuous in the connective tissue of the gonad, closely associated with branches of the visceral nerve. At points where they are in contact the nerve fibres are packed with secretory granules.

The cells which make this network are similar in appearance to the sheath cells described by Baxter & Nisbet (1963) in *Archachatina*. The elongated cell bodies attain a maximum size of $7 \times 2\mu$m. The cytoplasm is filled with electron-dense vesicles ranging in size from 0.5 to 1.0μm. There were no indications of different phases of secretory activity in the specimens examined.

DISCUSSION

One of the most difficult tasks in a study of neurosecretion using the light microscope is identification of NSM. The severe limitations of conventional staining when applied

to *Bithynia* are indicated in the foregoing account. Only 2 types of NSM were recognizable in paraffin sections, whereas 3 can be distinguished in electron micrographs, and another type of secretion, not separable from NSM by light microscopy, bears no resemblance to it at the ultrastructural level. The number and variety of pigmented inclusions in all neurones of *Bithynia,* as in other prosobranchs, also complicates interpretation of paraffin sections, but the pigmented inclusions are easily distinguished from NSM in electron micrographs.

It is known that the presence of neurosecretory granules is not in itself sufficient evidence to establish the neurosecretory nature of a neurone (Simpson, Bern & Nishioka, 1966), a point which is reinforced by the findings in *Bithynia*. Many neurones contain electron-dense granules, 87 to 93 nm in diameter, but they are not selectively stained, nor do their axons terminate in a neurohaemal area. This suggests that secretions of these neurones might incorporate neurotransmitters, rather than neurosecretory substances in the strict sense (neurohormones). Similar granules, thought to contain neurotransmitters have been described in "ordinary" neurones of *Lymnaea* (Boer,

Plate Explanation

Figure *3*: An "ordinary" neurone with cytoplasmic inclusions containing a yellow pigment thought to be carotenoid. Secretory granules are particularly evident near the Golgi bodies. $\times$ 25 000

Figure *4*: An "ordinary" neurone with lysosome-like bodies thought to contain lipofuscins. Secretory granules and vesicles with less dense contents are present. $\times$ 25 000

Figure *5*: A phloxinophil NS cell (type S1) from the cerebral ganglia. Immature neurosecretory granules occur in the vicinity of the numerous Golgi bodies. $\times$ 18 750

Figure *6*: An early secretory phase of a mucoid cell (type S4) of the left pleural ganglion. The cytoplasm contains not only secretory granules of the type seen in neurones, but also larger vesicles of mucoid secretion which arise as swellings on the granular endoplasmic reticulum. $\times$ 25 000

c – membrane-bounded inclusions thought to contain carotenoid pigment
cv – clear vesicle   fm – membrane devoid of ribosomes
gb – golgi body   ger – granular endoplasmic reticulum
gly – glycogen   ing – immature neurosecretory granule
li – lipid   ly – lysosome (= inclusion containing lipofuscin pigment)
mi – mitochondrion   n – nucleus   nf – neurofibril
nt – neurotubule   r – ribosome   sg – secretory granule
ser – smooth endoplasmic reticulum
sw – swelling on endoplasmic reticulum   tu – tubule

Plate Explanation

Figure *7*: An oblique section through the sub-oesophageal part of the visceral loop, showing a late secretory phase of a cell type S4. The cytoplasm is filled with secretory vesicles, some of which have burst. A fibre arising from the cell is devoid of vesicles. $\times$ 12 500

Figure *8*: Cytoplasmic detail of a similar cell with large areas of secretion within the cisternae of the granular endoplasmic reticulum. There are indications that vesicles with similar contents arise by

being pinched off from the cisternae. $\times$ 72 000

Figure *9*: A neurohaemal area of the sub-oesophageal ganglion, showing axones of NS cells intermingling with fibres of cells of type S4. They terminate against the perineurium, which contains blood spaces. $\times$ 6 250

Figure *10*: Fibres of cells of type S4 filled with secretion terminating against the perineurium and showing a pore-like area in the cell membrane. $\times$ 18 750

bm – basement membrane   bs – blood space   f – fibre
ger – granular endoplasmic reticulum   h – haemocoel
mi – mitochondrion   n – nucleus   npl – neuropile
NSM – neurosecretory material   p – pore   pn – perineurium
r – ribosome   rm – ruptured membrane   s – secretion
sg – secretory granule   tu – tubule   v – vesicle

Figure 3

Figure 4

Figure 5

Figure 6